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Veterinary Immunology and Immunopathology 105 (2005) 221–234
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Johne's disease in cattle is associated with enhanced expression of genes encoding IL-5, GATA-3, tissue inhibitors of matrix metalloproteinases 1 and 2, and factors promoting apoptosis in peripheral blood mononuclear cells

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Abstract

Infection of ruminants with *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) leads to a chronic and often fatal granulomatous enteritis known as Johne's disease. Most infections with *M. paratuberculosis* occur during the first 6 months of life, and there is some evidence for transmission in utero. Once established, infections typically exist in a subclinical state for several years. Recent gene-expression profiling studies suggested the hypothesis that inherent gene-expression profiles in peripheral blood mononuclear cells (PBMCs) from *M. paratuberculosis*-infected cattle may be different than expression profiles in PBMCs from uninfected controls. If true, this would suggest that it is possible to identify an *M. paratuberculosis* infection ''signature'' through transcriptional profiling of peripheral immune cells. In addition, identification of groups or classes of genes showing inherently different expression in PBMCs from *M. paratuberculosis*-infected cattle relative to PBMCs from uninfected controls might highlight important interactions between this pathogen and the host immune system. In this report, we describe studies aimed at testing this hypothesis. Our novel results indicate that, indeed expression profiles of at least 42 genes are inherently different in freshly isolated PBMCs from *M. paratuberculosis*-infected cattle when compared to similar cells from uninfected controls. Gene-expression differences observed following microarray analysis were verified and expanded upon by quantitative real-time PCR (Q-RT-PCR). Our results indicate that T cells within PBMCs from *M. paratuberculosis*-infected cows have adopted a predominant Th 2-like phenotype (enhanced expression of IL-5, GATA 3, and possibly IL-4 mRNA), that cells within infected cow PBMCs may exhibit tissue remodeling deficiencies through higher expression of tissue inhibitor of matrix metalloproteinase (TIMP) 1 and TIMP2 RNA and lower expression of matrix metalloproteinase (MMP) 14

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0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2005.02.009

RNA than similar cells from healthy controls, and that cells within the PBMC population of *M. paratuberculosis*-infected cows are likely poised for rapid apoptosis (upregulation of CIDE-A, Bad, TNFRI, and Fas).

Keywords: Paratuberculosis; Johne's disease; Gene expression; Microarray; Functional genomics

1. Introduction

Johne's disease is a granulomatous enteritis of ruminants caused by the intracellular bacterium *Mycobacterium avium*, subspecies *paratuberculosis* (*M. paratuberculosis*). Infections with *M. paratuberculosis* typically occur in the first 6 months of life and can persist for up to several years with few outward signs of infection (Merkal et al., 1975; Kennedy and Benedictus, 2001; Manning and Collins, 2001; Storset et al., 2001).

As with other intracellular bacterial infections, control of *M. paratuberculosis* relies on maintenance of a T helper (Th) 1-like or pro-inflammatory and cytotoxic immune response (Munk and Emoto, 1995; Silva et al., 1996; Kaufmann, 1999; Sousa et al., 2000). Indeed, presentation of clinical symptoms, including chronic diarrhea, wasting, and eventually death are often preceded by a transition in the predominant immune response from Th 1-like to Th 2-like (Navarro et al., 1998; Perez et al., 1999; Stabel, 2000). Lesions associated with *M. paratuberculosis* infection are generally restricted to the small intestine, particularly the ileum and ileal–cecal junction, although more disseminated infections do occur (Buergelt et al., 1978; Clarke and Little, 1996; Whitlock and Buergelt, 1996). Lesions are characterized by large numbers of infected macrophages containing acid-fast bacilli, high expression of interleukin (IL)-1α and other pro-inflammatory cytokines, thickening of the ileal wall, and chronic inflammation (Whitlock and Buergelt, 1996; Aho et al., 2003). In most cases, mesenteric lymph nodes draining sites of infection exhibit hyperplasia and also contain acid-fast staining macrophages and dendritic cells (Koets et al., 2002). The long-term chronic nature of infections with *M. paratuberculosis*, involvement of the extensive gut lymph system, and transitions in peripheral immune responses over time all suggest that infections with *M. paratuberculosis* might have a profound effect on host immune cell gene-expression profiles.

Indeed, gene-expression profiling of peripheral blood mononuclear cells (PBMCs) from *M. paratuberculosis*-infected and control cows revealed that in vitro stimulation with *M. paratuberculosis* induced profound, positive, but short-lived changes in immune cell gene expression in cells from infected cattle compared to cells from control cattle (Coussens et al., 2004). Longer term stimulation (>18 h) resulted in repressed expression of many genes (Coussens et al., 2002, 2004). Furthermore, many differences in immune cell gene expression were evident between groups of infected and control cows cultured overnight in the absence of *M. paratuberculosis* stimulation (nil treatment) (Coussens et al., 2003a,b).

These results led to the hypothesis that *M. paratuberculosis* infection alters host immune cell gene-expression profiles, and thus, the inherent gene-expression profile of freshly isolated PBMCs from infected cattle will be detectably different from that of healthy controls. In this report, we describe experiments designed to test this hypothesis directly. Our novel results indicate that, indeed, differences in gene-expression profiles of freshly isolated PBMCs from *M. paratuberculosis*-infected and healthy control cows are different. In addition, our results reveal an inherent Th 2-like gene-expression pattern in PBMCs from *M. paratuberculosis*-infected cows as well as expression of genes suggesting a population of immune cells in PBMCs from infected cows is poised for rapid induction of apoptosis by both internal and external signals.

2. Materials and methods

2.1. Experimental animals and preparation of PBMCs

To ensure results of our study were broadly representative, infected and control Holstein cows were obtained from two separate groups. Group 1

consisted of four cows naturally infected with *M. paratuberculsosis* and four healthy uninfected controls from a commercial herd in Michigan (USA). Group 1 cows were used in both microarray and quantitative real-time PCR (Q-RT-PCR) experiments. Group 2 consisted of six cows naturally infected with *M. paratuberculosis* and four healthy uninfected controls obtained from commercial dairy operations in Denmark. Group 2 cows were used only for Q-RT-PCR validation of selected differentially expressed genes. All experimental cows were between 24 and 48 months of age and were extensively tested for presence of serum antibodies to *M. paratuberculosis* (Herd Check ELISA, IDEXX Corp.) and IFN-γ response of whole blood exposed to *M. paratuberculosis* antigens. Results of fecal culture testing revealed intermittent and low-level bacterial shedding (<100 CFU/g feces) in most infected cows.

Blood samples (approximately 100 ml) were obtained from all animals via either the jugular or coccygeal (tail) vein using 2.5-cm 21-gauge needles and were collected into 8-ml Vacutainer tubes containing acid-citrate dextrose (ACD) as an anticoagulant (BD Vacutainer, Rutherford, NJ). PBMCs from Group 1 animals were prepared as previously described (Coussens et al., 2002, 2004). Briefly, blood samples were centrifuged at 4 °C for 30 min at 1800 rpm. The resulting buffy coats were transferred to a new 50-ml conical tube containing 32 ml of cold sterile phosphate-buffered saline (PBS) overlaid on a 10-ml cushion of Percoll (1.084 g/ml; Sigma Chemical Co. St. Louis, Mo). Typically, buffy coats from three to four Vacutainer tubes from a single animal were added to each Percoll gradient. Cells were then centrifuged at 1380 rpm for 40 min at room temperature to separate erythrocytes and polymorphonuclear leukocytes from mononuclear cells. Following careful aspiration of PBS, PBMCs at the PBS–Percoll interface were transferred to new 50-ml conical tubes and rinsed with 20 ml of cold sterile PBS. Final cell pellets were collected by centrifugation for 5 min at 1800 rpm at 4 °C. Finally, the PBS was aspirated, cells were briefly vortexed to loosen clumps, and 4 ml of Trizol reagent (Invitrogen) was added to disrupt cells and preserve RNA. Trizol preparations were frozen at −80 °C until use. Total leukocytes from Danish cows (Group 2) were prepared by hypotonic lysis and hypertonic restore following isolation of buffy coats as described above. Total leukocytes were then frozen at −80 °C in Trizol and transported to the Center for Animal Functional Genomics at Michigan State University on dry ice by overnight courier.

2.2. RNA extraction, preparation of labeled cDNA, and microarray hybridization

RNA was extracted from PBMCs in Trizol reagent (Invitrogen Inc., Carlsbad, CA) as recommended by the manufacturer. The quantity and quality of the extracted total RNA was estimated by UV spectrophotometry and electrophoresis on 1% native agarose gels. Quality and integrity of RNA was further evaluated using an Agilent 2100 Bioanalyzer and the RNA Nanochip essentially as recommended by the manufacturer (Agilent Technologies, Palo Alto, CA).

To evaluate gene-expression profiles of PBMCs from Group 1 cows, total RNA (8 μg) from each cow was used as template in reverse transcription reactions (Atlas Powerscript labeling system; BD Biosciences Inc., Alameda, CA) in which oligo$(dT)_{15–18}$ was used as primer (Coussens et al., 2002, 2003a). In the Atlas Powerscript system, cDNA is prepared with a randomly incorporated amino-modified dUTP. To provide a control for cDNA synthesis and labeling efficiency, as well as for subsequent cDNA microarray hybridization, 650 pg of synthetic lambda Q gene RNA containing an engineered poly(A) tail was spiked into each cDNA synthesis reaction mixture.

Following first-strand synthesis, cDNAs from randomly paired control and infected cows were differentially labeled using *N*-hydroxysuccinimide-derivatized Cy3 and Cy5 dyes (Amersham Pharmacia, Ltd., Piscataway, NJ). A dye swap design was used to offset potential dye bias, resulting in two infected cow cDNAs labeled with Cy3 each being directly compared within a microarray with one of two Cy5-labeled control cow samples and vice versa. Labeled cDNAs were purified to remove unincorporated dyes using cDNA labeling purification modules (Invitrogen Inc.), as previously described (Coussens et al., 2002, 2003a). Differentially labeled samples (infected versus control) were then combined and concentrated to 10 μl by using Microcon 30 spin concentrators (Millipore Corp., Bedford, MA). Microarray hybridization was performed after addition of

100 μl of SlideHyb-3 (Ambion Inc., Alameda, CA) to the concentrated Cy3–Cy5-labeled probe cDNAs.

Microarray hybridizations were conducted for 18 h in a commercial microarray hybridization station by using a step-down hybridization protocol (GeneTAC; Genomics Solutions Inc., Ann Arbor, MI) as described previously (Coussens et al., 2003a, 2004). BOTL-3 bovine cDNA microarrays from the Center for Animal Functional Genomics (CAFG) at Michigan State University were employed in this study. These microarrays contain a combination of approximately 750 expressed sequence tag clones from a bovine total leukocyte cDNA library and 500 PCR amplicons representing genes known to function in immune response and have been described previously (Coussens et al., 2003a, 2004). All genes on the BOTL-3 microarray are spotted in triplicate. The full list of genes represented on BOTL-3 microarrays as well as other annotation can be found at www.cafg.msu.edu.

Following hybridization, cDNA microarrays were washed in the hybridization station, rinsed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and once in double-distilled H_2O, and finally dried by centrifugation in a cushioned 50-ml conical centrifuge tube. This process yielded BOTL-3 cDNA microarrays, which allowed direct comparison of PBMC gene expression between four separate control and four separate infected cows.

Final hybridized microarrays were scanned by using a GeneTAC LS IV microarray scanner and GeneTAC LS software (Genomic Solutions Inc.). Gene TAC analyzer software was then used to process microarray images, find spots, integrate robot-spotting files with the microarray image, and finally to create reports of raw total spot intensities for both dyes. Raw microarray data used in this report can be found under the ''Links'' Section of the CAFG website (www.cafg.msu.edu).

2.3. Microarray data analysis

Raw total intensity values for each spot on the microarray were converted to comma separated values files, transferred to Excel spreadsheets and prepared for LOWESS normalization using PROC LOESS from the statistical software package SAS (SAS, 1990; Cleveland and Grosse, 1991; SAS Institute, 2000). Success of LOWESS normalization was monitored by plotting log intensity ratio (M = log Cy3 − log Cy5) versus the mean log intensities (A = ((log Cy3 + log Cy5)/2)) for each microarray (Yang et al., 2002). LOWESS normalized microarray data were subsequently processed essentially as described (Coussens et al., 2002, 2003a, 2004). Briefly, the three gene replicate spots on each BOTL-3 microarray were combined to yield a mean LOWESS normalized intensity value for each gene. Mean LOWESS normalized values ($\log_{10}$) were back transformed and the median negative value for each dye within array was subtracted to account for background intensities. Background corrected values were re-transformed (natural log, ln) and used to calculate ln difference values (infected versus mean control value) for each gene represented on the BOTL-3 microarray. This process restricts analysis to only those genes expressed at levels clearly distinguishable from background, since those whose intensity is below background levels result in negative values and cannot be log transformed. Finally, ln difference values were combined across the four biological replicates in this study to yield a final mean ln difference, standard error, T-STAT, and T-distribution for each gene. Anti-logs of mean ln differences were used as an approximation of fold change. Final data were then filtered to highlight those genes with a fold change of >1.5-fold up or down and $P < 0.05$.

2.4. Validation of gene-expression differences by quantitative real-time PCR

Validation of selected gene-expression changes observed on cDNA microarrays was performed by quantitative real-time PCR, using an Applied Biosystems 7000 DNA sequence detection system (Perkin-Elmer Corp., Foster City, CA) and the fluorescent dye SYBR Green, as described previously (Coussens et al., 2003b). Briefly, total RNA extracted from PBMCs of *M. paratuberculosis*-infected ($n = 4$), and control uninfected cows ($n = 4$) from Group 1 (US herd), and Group 2 (Danish herds: infected, $n = 6$; control, $n = 4$) were converted into first-stand cDNA essentially as described (Coussens et al., 2003b).

Reverse transcription reactions were allowed to progress at 42° for 60 min at which time reactions were heated to 70° for 5 min and then cooled to 37° prior to addition of 2 U of DNase-free RNase H

(Invitrogen Inc.). Reactions were incubated at 37° for 20 min in the presence of RNase H to remove original RNA templates. RNase H was subsequently inactivated by adding 0.1 μM of EDTA and heating to 70 °C for 10 min. First strand cDNAs were precipitated in ethanol with 3 M sodium acetate and final cDNA pellets were suspended in DNase–RNase-free ddH_2O. Final cDNA samples were diluted to 10 ηg/μl based on UV spectrophotometric analysis. All dilutions were stored at −80 °C until they were used in Q-RT-PCR reactions.

Q-RT-PCR was performed by using SYBR Green PCR master Mix (Perkin-Elmer Corp., Palo Alto, CA), 25 ηg of template cDNA, and gene-specific primers. All primers were designed using Primer Express software based on sequences obtained from the CAFG database, NCBI Genbank, or the TIGR bovine gene index (bTGI) database. Primers were synthesized at a commercial facility (Operon Technologies, Alameda, CA). All reactions were run in triplicate, and data were analyzed using the $2^{-(\Delta\Delta Ct)}$ method as described previously (Livak and Schmittgen, 2001; Coussens et al., 2003b). To assess differential gene expression between groups, β-actin was used as the control gene, and the mean control cow ΔCt value was used as the calibrator. Triplicate sample values within cow were combined to produce a mean ΔΔCt value for each animal and then these values were combined within group to produce an overall group mean ΔΔCt value. Significance was determined by Student's *t*-test and expression differences considered significant when $P < 0.05$.

3. Results

3.1. Summary of microarray data

Following normalization and analysis of microarray data as described in Section 2, data were filtered to reveal genes with expression differences (infected versus control cows) of greater then 1.5-fold and $P < 0.05$ across the four initial biological replicates. Of the ∼1200 individual genes represented on BOTL-3 microarrays, 42 were identified as significantly differentially expressed in freshly isolated PBMCs from infected and control cows under these selection criteria. In contrast, the experimentally determined background (number of genes showing differential expression when, in fact, there should be no difference) on a microarray of this size, following LOWESS normalization and given the criteria of 1.5-fold change and $P < 0.05$ is approximately four genes (Nobis et al., 2003). A list of all 42 differentially expressed genes, with annotation, has been made available as supplemental information at www.cafg.msu.edu under the ''Links'' Section. Of the 42 significantly differentially expressed genes, only 7 genes were shown to be expressed at lower levels in PBMCs from *M. paratuberculosis*-infected cows relative to control uninfected cows. The greatest fold differences occurred in expression of genes encoding the TNFα converting enzyme ADAM 17 (15-fold), the BCL-2-binding protein Bad (11-fold), poly(ADP-ribose) polymerase (PARP) (8-fold), MCL1 (3.4-fold), a 14-3-3 protein family member (2.6-fold), and TRAF1 (2.5-fold).

Differentially expressed genes were annotated using a web-accessible database, GeneLink (www.cafg.msu.edu), which contains extensive information on each gene represented on the BOTL-3 cDNA microarray, including a summary of protein function (if known), links to PubMed references describing the gene or its encoded protein, metabolic pathway information, and gene ontology. When genes were grouped into major classifications based on known function, cytokines and their receptors comprised 14.3% of differentially expressed genes (six genes), matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), and other proteins involved in cell migration or tissue remodeling comprised 21.4% of differentially expressed genes (nine genes), and apoptosis and signal transduction proteins comprised 19% of differentially expressed genes (eight genes). In total, these three classifications accounted for over 50% of genes differentially expressed in freshly isolated PBMCs from *M. paratuberculosis*-infected cows, relative to PBMCs from uninfected controls.

3.2. Differential expression of cytokine genes

Microarray analysis revealed that the gene encoding Interleukin (IL)-5 was expressed over five-fold higher in PBMCs from *M. paratuberculosis*-infected cows, relative to similar cells from uninfected cows

Table 1
Cytokine genes differentially expressed in PBMCs from *M. paratuberculosis* infected and control uninfected cattle

Gene	*P*-value	Fold change	Ontology
IL-5	0.00137	5.4	Cytokine
TGFβ	0.00732	3.4	Cytokine
GM-CSF	0.00572	5.2	Cytokine
Activin A receptor IB	0.00291	2.9	Receptor
Lymphotactin	0.00838	5.4	Cytokine
Tie 2	0.01666	5.0	Receptor

(Table 1). Since both IL-5 and IL-4 are cytokines typically associated with a Th 2-like T cell phenotype (Agnello et al., 2003), we re-examined our microarray data for IL-4 gene-expression differences. RNA-encoding IL-4 was, in fact, expressed over three-fold higher in PBMCs from *M. paratuberculosis*-infected cows relative to PBMCs from uninfected controls. However, IL-4 gene expression was quite variable and did not survive our initial screen because the significance of this gene-expression difference ($P = 0.169285$) was below our cut-off value of $P < 0.05$.

Upregulation of IL-5 and possibly IL-4 gene expression in PBMCs from *M. paratuberculosis*-infected cows suggested that the T cell population in PBMCs from these cows may have a higher proportion of cells with a Th 2-like phenotype than is found in PBMCs from uninfected controls. To examine this hypothesis, we next conducted Q-RT-PCR analysis examining the expression of two transcription factors (not represented on the BOTL-3 microarray) known to polarize T cells towards the Th 1 (T-bet) or Th 2 (GATA 3) phenotype (Dong and Flavell, 2000; Rao and Avni, 2000; Szabo et al., 2000; Agnello et al., 2003). Indeed, freshly isolated PBMCs from *M. paratuberculosis*-infected cows consistently expressed higher levels of RNA encoding the Th 2 polarizing transcription factor GATA 3 than PBMCs from uninfected controls (Fig. 1). Conversely, there was little or no difference in expression of RNA encoding the Th 1 polarizing transcription factor T-bet between infection groups (Fig. 1). Data presented in Fig. 1 represent cows from both Groups 1 and 2 (infected, $n = 10$–16; uninfected, $n = 6$–10), suggesting that the trend toward a higher proportion of Th 2-polarized T cells in freshly isolated PBMCs from *M. paratuberculosis*-infected cows is a general phenomena, not restricted to a single group, geographical location, or management scheme.

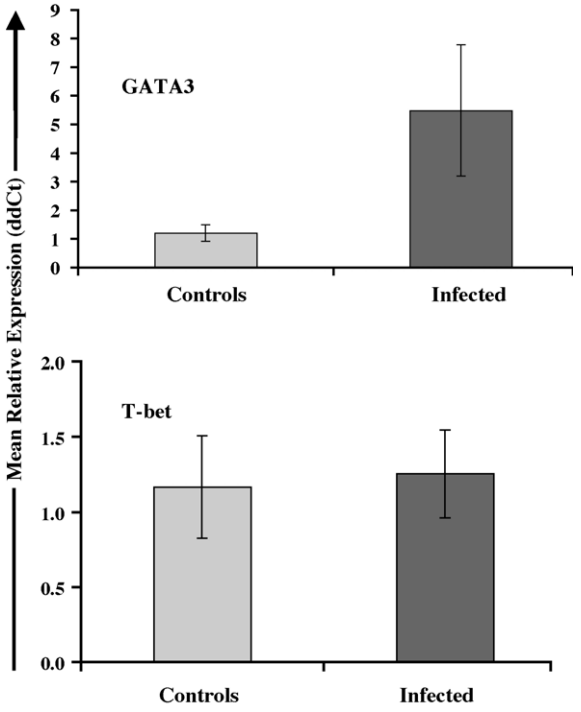

Fig. 1. Expression of RNA-encoding GATA-3 and T-bet transcription factors in freshly isolated PBMCs from cattle infected with *M. paratuberculosis* (GATA-3, $n = 8$; T-bet, $n = 9$) and from healthy control cattle (GATA-3, $n = 6$; T-bet, $n = 9$). RNA abundance was determined by Q-RT-PCR as described in Section 2. Data from the Q-RT-PCR assay were analyzed using the $2^{-\Delta\Delta Ct}$ method as described (Livak and Schmittgen, 2001; Coussens et al., 2003). In this analysis, β-actin served as the control gene to calculate ΔCt for each sample. The mean ΔCt value for all controls served as the calibrator for calculation of the final $2^{-\Delta\Delta Ct}$ value for each sample. Data from each animal were combined within infection group to calculate a mean relative expression value within group and a standard error of the mean (S.E.M., error bars).

Additional cytokines found to be differentially expressed in PBMCs from *M. paratuberculosis*-infected cows and uninfected controls, based on microarray analysis, included transforming growth factor-β (TGFβ) (3.4-fold higher in PBMCs from infected cows relative to controls) and granulocyte macrophage colony stimulating factor (GM-CSF) (5.2-fold higher in PBMCs from infected cows relative to controls). TGFβ is an immune modulating cytokine that can limit pro-inflammatory responses and has profound effects on tissue remodeling via regulation of matrix metalloproteinases and their

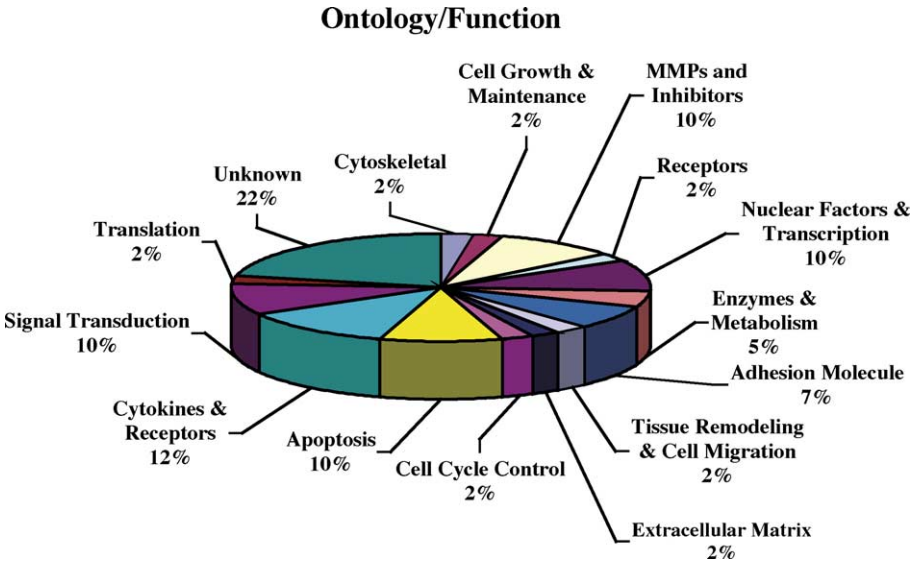

Fig. 2. Major ontological and functional classification of genes found to be significantly differentially expressed (>1.5-fold; $P < 0.05$) in freshly isolated PBMCs from cattle infected with *M. paratuberculosis* relative to cells from healthy controls. Following microarray processing and data analysis as described in Section 2, the resulting gene list was uploaded into the gene name search window of a web accessible database (GeneLinks, www.cafg.msu.edu). GeneLinks returns an HTML page containing extensive information on each gene and the protein product it encodes (if known). Ontology and/or function was derived by evaluated both the information from Amigo links within the GeneLink HTML page and from the gene product description, which is derived from the LocusLink match from each gene on the BOTL-3 microarray.

inhibitors (Cotton et al., 1998; Li et al., 2000; Siwik and Colucci, 2004).

3.3. Differential expression of matrix metalloproteinases and their inhibitors

Microarray analysis of gene expression in PBMCs from *M. paratuberculosis*-infected and uninfected control cows suggested that several genes encoding the MMPs and TIMPs were significantly differentially expressed in these two cell populations (Fig. 2). Primary among these genes were those encoding TIMPs 1, 2 and 3 and MMPs 14 and 15 along with ADAM17 (Table 2). These data are consistent with known effects of TGFβ and IL-4, with TGFβ known to upregulate expression of TIMP1, TIMP2, and TIMP3, and IL-4 known to upregulate TIMP2 (Edwards et al., 1987; Ihn et al., 2002; Seeland et al., 2002). In addition, these results are consistent with previous observations indicating that *M. paratuberculosis* has a profound effect on expression of several MMP genes, including MMP9, MMP14, and MMP23 in PBMCs from infected cows exposed to live *M. paratuberculosis* in vitro (Coussens et al., 2002, 2003a).

In order to verify differential expression of genes encoding the various TIMPs observed as differentially expressed on cDNA microarrays, Q-RT-PCR was conducted as described in Section 2 using gene-specific primers. When data were combined across both Groups 1- and 2-infected cows, relative to their appropriate control uninfected cows, mean relative expression of the TIMP1 gene was over three-fold

Table 2
Differential expression of gene encoding matrix metalloproteinases and their inhibitors in PBMCs from *M. paratuberculosis* infected cattle relative to PBMCs from control uninfected cattle

Gene	*P*-value	Fold change	Ontology
TIMP-1	0.04792	4.8	MMPs and inhibitors
TIMP2	0.01661	3.3	MMPs and inhibitors
TIMP3	0.052053	11.1	MMPs and inhibitors
MT1-MMP (MMP14)	0.04068	−1.5	MMPs and inhibitors
MMP15	0.03392	2.7	MMPs and inhibitors
ADAM17/TACE	0.04002	15.8	MMPs and inhibitors

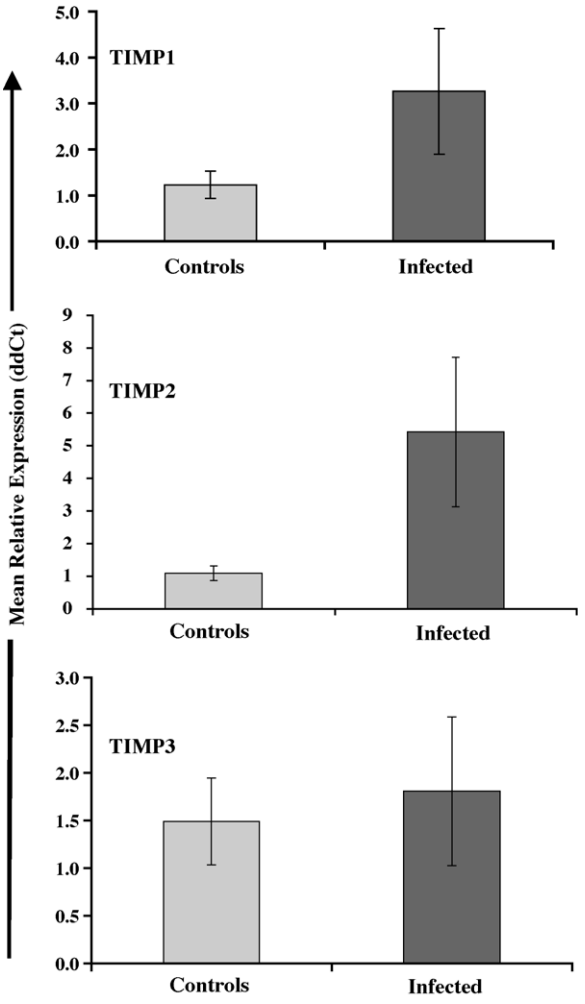

Fig. 3. Expression of RNA-encoding TIMP1, TIMP2, and TIMP3 in freshly isolated PBMCs from cattle infected with *M. paratuberculosis* (TIMP1, $n = 4$; TIMP2, $n = 7$; TIMP3, $n = 7$) and healthy control cattle (TIMP1, $n = 4$; TIMP2, $n = 5$; TIMP3, $n = 6$) as determined by Q-RT-PCR. RNA abundance was determined by Q-RT-PCR as described in Section 2. Data from the Q-RT-PCR assay were analyzed as described in the legend for Fig. 1. Data from each animal were combined within infection group to calculate a mean relative expression value within group and a standard error of the mean (S.E.M., error bars).

higher in PBMCs from infected cows then in similar cells from control cows (Fig. 3). Likewise, mean relative expression of TIMP2 was over five-fold higher in PBMCs from infected cows than in PBMCs from uninfected controls (Fig. 3). In contrast, Q-RT-PCR failed to verify enhanced expression of TIMP3 in PBMCs from *M. paratuberculosis*-infected cows relative to controls (Fig. 3).

3.4. Expression of genes encoding proteins with known roles in apoptosis and signal transduction

Another major group of genes revealed as differentially expressed in microarray experiments comparing PBMCs from *M. paratuberculosis*-infected versus uninfected control cows encoded proteins involved in apoptosis and signal transduction (Fig. 2). The eight genes in this classification were examined and annotated as to their involvement in either apoptosis induction (Pro) or inhibition of apoptosis (Anti) (Table 3). In the case of IGFBP6, a clear determination could not be made (ND). In total, three genes were scored as being anti-apoptotic and the remaining four were scored as pro-apoptotic (Table 3). All three genes scored as anti-apoptotic were expressed at significantly lower levels in PBMCs from *M. paratuberculosis*-infected cows, relative to PBMCs from uninfected control cows (Table 3). Conversely, all four genes scored as pro-apoptotic were expressed at significantly higher levels in PBMCs from infected cows than in similar cells from control uninfected cows (Table 3).

Of the pro-apoptotic genes listed in Table 3, Bad (a pro-apoptotic BCL-2 family member), and CIDE-A (a member of the DNA fragmentation factor family) are some of the most well known and best characterized in terms of a role in induction of apoptosis (Chao and Korsmeyer, 1998; Inohara et al., 1998). When expression of these genes was evaluated by Q-RT-PCR in PBMCs from *M. paratuberculosis*-infected and control cows from both study groups, (Groups 1 and 2), relative to their appropriate controls, both genes were verified as being expressed at significantly higher levels in PBMCs from infected cows relative to similar cells from uninfected controls (Fig. 4).

Based on our microarray and Q-RT-PCR observations suggesting up regulation of several genes encoding pro-apoptotic proteins, we re-examined our original microarray data, reducing the stringency of selection to ± 1.5-fold (up or down regulated in infected relative to control cows) and $P < 0.15$. This reduced stringency indicated up regulation of several additional apoptosis related genes, including

Table 3
Apoptosis and signal transduction genes differentially expressed in PBMCs from *M. paratubercuosis* infected cattle and PBMCs from uninfected control cattle

Gene or clone name	*P*-value	Fold change	Classification	Pro or anti apoptotic
IGFBP6	0.00150	6.1	Signal transduction	ND
MCL1 (MUSK)	0.00237	−3.4	Apoptosis	Anti
BOTL0100008_E01 (WD40 repeat containing protein)	0.00552	1.5	Signal transduction	Likely Pro
Somatic cytochrome *c*	0.01001	3.2	Apoptosis	Pro
Cell death activator CIDE-A	0.01162	2.5	Apoptosis	Pro
BOTL0100001XH09R (A 14-3-3 family member)	0.01580	−2.6	Signal transduction	Anti
Bcl-2 binding component 6 (Bad)	0.03617	11.9	Apoptosis	Pro
TRAF1	0.04794	−2.5	Signal transduction	Anti

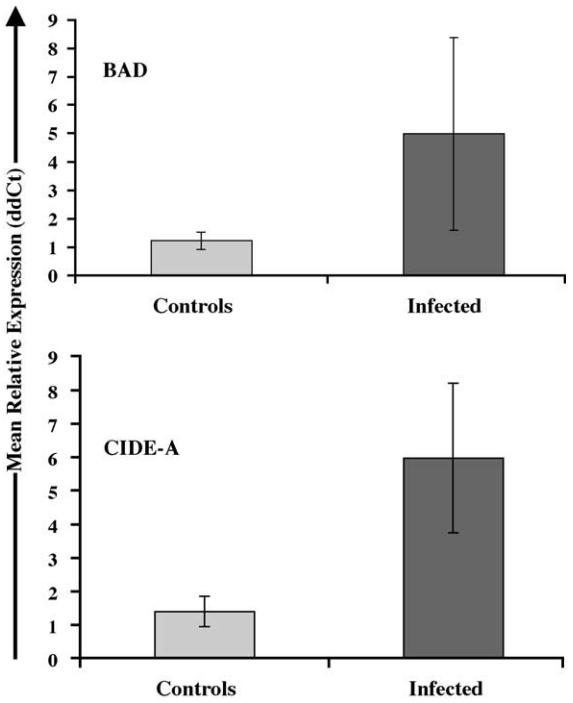

Fig. 4. Expression of RNA-encoding BAD and CIDE-A in freshly isolated PBMCs from cattle infected with *M. paratuberculosis* (BAD, $n = 9$; CIDE-A, $n = 7$) and healthy control cattle (BAD, $n = 4$; CIDE-A, $n = 6$) as determined by Q-RT-PCR. RNA abundance was determined by Q-RT-PCR as described in Section 2. Data from the Q-RT-PCR assay were analysed. Data from the Q-RT-PCR assay were analyzed as described in the legend for Fig. 1. Data from each animal were combined within infection group to calculate a mean relative expression value within group and a standard error of the mean (S.E.M., error bars).

those encoding TNF receptor 1 (TNFR1) (2.4-fold), the 14-3-3 protein (1.5-fold), RIP kinase (3.1-fold), Fas (CD95) (4.2-fold), and TRADD (1.9-fold). Of particular interest in this additional group of genes were those encoding CD95 and TNFR1, both representing potential external mechanisms of cell death induction. Q-RT-PCR was used to confirm expression levels of these two TNF receptor superfamily members using gene-specific primers. Consistent with microarray data, expression levels of genes encoding both TNFR1 and CD95 were elevated in PBMCs from *M. paratuberculosis*-infected cows, relative to PBMCs from uninfected controls (Fig. 5).

4. Discussion

Gene-expression profiling has previously been employed to investigate gene-expression changes in PBMCs from *M. paratuberculosis*-infected cattle following in vitro stimulation with *M. paratuberculosis* (Coussens et al., 2002, 2003a, 2004), differences in gene expression between intestinal tissues from *M. paratuberculosis*-infected and control uninfected cattle (Aho et al., 2003), gene-expression differences in mesenteric lymph nodes of *M. paratuberculosis*-infected and control uninfected cattle (McNulty et al., in preparation), and in macrophages exposed to *M. paratuberculosis* in vitro (Tooker et al., 2002; Tooker and Coussens, 2004). These studies have shed considerable light on the host immune response to

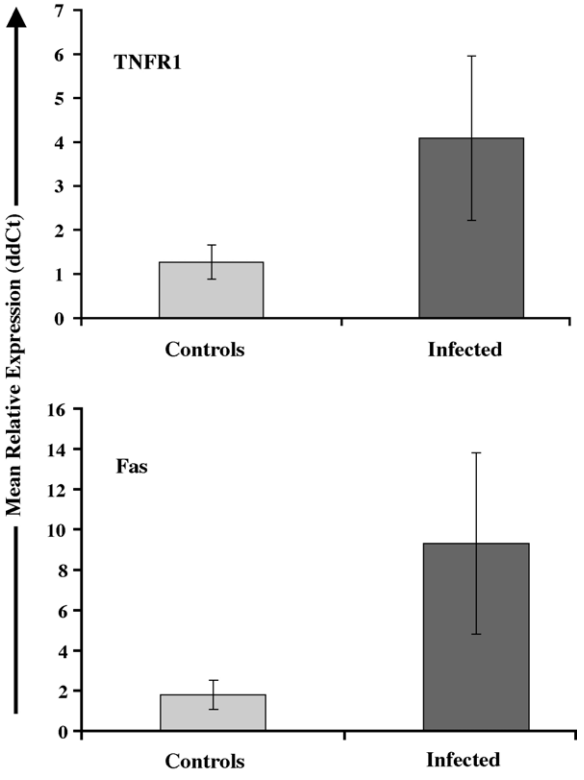

Fig. 5. Expression of RNA-encoding TNFR1 and Fas (CD95) in freshly isolated PBMCs from cattle infected with *M. paratuberculosis* (TNFR1, $n = 7$; Fas, $n = 6$) and healthy control cattle (TNFR1, $n = 6$; Fas, $n = 5$) as determined by Q-RT-PCR. RNA abundance was determined by Q-RT-PCR as described in Section 2. Data from the Q-RT-PCR assay were analysed. Data from the Q-RT-PCR assay were analyzed as described in the legend for Fig. 1. Data from each animal were combined within infection group to calculate a mean relative expression value within group and a standard error of the mean (S.E.M., error bars).

M. paratuberculosis and have led to formulation of novel hypotheses regarding these interactions (Coussens, 2004). One such hypothesis was that there would be inherent gene-expression differences in PBMCs from *M. paratuberculosis*-infected cattle and similar cells from control uninfected cattle.

In the present study, we have tested this hypothesis by comparing expression levels of ~1200 genes in freshly isolated PBMCs and total leukocytes from *M. paratuberculosis*-infected cows to expression in similar cells from uninfected control cows. Comparisons were made using a "boutique" bovine cDNA microarray, BOTL-3, which contains many genes known to function in immune responses as well as numerous randomly selected ESTs from a bovine total leukocyte cDNA library (Coussens et al., 2003a). This microarray has been used extensively in studies of immune response, reproduction, and innate immunity (Coussens et al., 2003a, 2004; Madsen et al., 2003; Evans et al., 2004). At this writing, the BOTL microarray, Version 5 (BOTL-5, ~1500 genes, including numerous controls) is now being produced and has been used to add to the data set discussed in this report. To date, over 10 direct comparisons have been made between freshly isolated PBMCs or total leukocytes from *M. paratuberculosis*-infected cows and similar cells from uninfected controls. These studies, focused on diagnostic indicators of infection, have highlighted over 50 significantly (>1.5-fold change; $P < 0.05$) differentially expressed genes (Skovgaard et al., in preparation).

Immune responses to *M. paratuberculosis* develop and change over a long course of chronic infection (typically 2–5 years). As infections are primarily restricted to the gut, mesenteric lymph nodes are the most likely site of *M. paratuberculosis* antigen presentation and development of the adaptive immune response. Given the long-term nature of *M. paratuberculosis* infections, the potency of many Mycobacterial antigens, and the extensive nature of the gut lymph system, it is reasonable to assume that infections with *M. paratuberculosis* will have a profound effect on circulating immune cells (Coussens, 2004). However, previous studies have shown that the relative proportions of various immune cells and their subsets in PBMCs of subclinically infected cows do not differ substantially from that of uninfected controls, as studied by flow-cytometry (Koets et al., 2002; Coussens et al., 2003a). Thus, any differences are likely to be found within the various immune cells at the gene or protein expression level. Indeed, our recent studies suggested that many differences in gene expression exist in nil stimulated and cultured PBMCs from *M. paratuberculosis*-infected cattle and those from healthy controls (Coussens et al., 2003a,b). However, this study relied on indirect comparisons using a mixed-model analysis, and it is reasonable to assume that placing cells in culture overnight would itself have profound effects on gene expression. Nevertheless, these earlier observations led directly to the hypothesis tested in the

current report, that inherent gene-expression profiles of freshly isolated PBMCs and total leukocytes from *M. paratuberculosis*-infected and control uninfected control cows would be different. Such differences could, therefore, be detected without the need for culture or antigen stimulation.

Data presented in this report support this novel hypothesis and demonstrate expression differences in several key groups of genes that would have a profound effect on immune cell response to antigens, migration of immune cells into infected tissues, and apoptosis of immune cell populations. Our data suggest that T cells within the PBMC population from *M. paratuberculosis*-infected cows are skewed toward a Th 2-like phenotype with high expression of genes encoding IL-5 and likely IL-4, in addition to GATA-3, a Th 2 polarizing transcription factor. All cows in this study were classified as subclinical, were ELISA positive for antibodies to *M. paratuberculosis*, produced enhanced levels of IFN-γ upon stimulation of whole blood with *M. paratuberculosis* antigens, and most cows were shedding only small amounts of *M. paratuberculosis* in feces (data not shown). The fact that all cows still demonstrated an IFN-γ response suggests that the outward immune response had not yet completely moved in favor of a Th 2-like response, as is observed in many clinically infected cattle (Stabel, 2000). However, the chronic presence of *M. paratuberculosis* was probably beginning to have an effect on the phenotype of circulating T cell populations. In a previous study, we demonstrated that cultured, but unstimulated PBMCs from subclinical *M. paratuberculosis*-infected cows, exhibited a gene-expression pattern consistent with a pro-inflammatory program, including elevated levels RNA-encoding IFNγ, IL-1α, and IL-6 (Coussens et al., 2003b). The combination of these two studies suggests that, indeed, culture of PBMCs without stimulation may have a dramatic impact on the gene-expression program of many immune cells. This might be expected because these cells would be producing various cytokines and other factors that gradually build up within the culture media and would have profound effects on gene and protein expression. A time course study has been initiated to resolve this issue. Furthermore, the present study relied on total PBMC populations and did not use well-characterized isolated immune cell subsets. Such studies are clearly warranted and should help extend our understanding of how a long-term chronic infection, such as Johne's disease affects individual immune cell populations.

Enhanced expression of the gene encoding TGFβ is of particular interest for several reasons, including the role of this cytokine in immune modulation and its role in regulating expression of MMPs and TIMPs. Previous studies demonstrated significantly elevated levels of IL-10 gene expression in *M. paratuberculosis* stimulated PBMCs from infected cattle, but not enhanced expression of TGFβ (Coussens et al., 2003b). In combination, these results suggest a hypothesis that a general regulatory cell population expressing TGFβ may exist in PBMCs from *M. paratuberculosis*-infected cows and could limit pro-inflammatory activity in a general manner. However, once pro-inflammatory cells are activated by antigen, at least in vitro, a regulatory T cell population responds by production of large amounts of IL-10. This novel hypothesis awaits testing. Importantly, neutralization of IL-10 by monoclonal antibodies causes elevated production of IFNγ by PBMCs from *M. paratuberculosis*-infected cattle following exposure to specific antigen, suggesting that IL-10 is in fact limiting IFNγ production by these cells (Buza et al., 2004).

In keeping with well-known linkages between TGFβ expression and MMP/TIMP gene expression (Cotton et al., 1998; Li et al., 2000; Siwik and Colucci, 2004), our initial microarray studies and subsequent Q-RT-PCR assays indicated that PBMCs from *M. paratuberculosis*-infected cows expressed far more TIMP1 and TIMP2 than PBMCs from uninfected controls. If this result translates to enhanced levels of TIMP1 and TIMP2 protein; then, it is likely that cells with enhanced TIMP expression would be deficient in tissue migration, a potentially serious defect, particularly considering the nature of *M. paratuberculosis* infections. If this expression pattern persists in infected tissues, then this may help explain the thickened and corrugated nature of *M. paratuberculosis*-infected intestinal tissue.

The final category of genes found to be differentially expressed in freshly isolated PBMCs from *M. paratuberculosis*-infected cows relative to PBMCs from uninfected controls that were considered in this report encode proteins known to be involved in

apoptosis and signal transduction. Our initial analysis of microarray data highlighted several genes encoding proteins involved in apoptosis, including CIDE-A, Bad, TRAF1, MCL1, cytochrome *c*, and a 14-3-3 family member. Q-RT-PCR was used to validate some of these gene-expression changes, particularly for genes encoding Bad and CIDE-A. Gene-expression differences observed from this initial analysis suggested that cells within the PBMC population from *M. paratuberculosis*-infected cows may be poised for rapid apoptosis, relative to similar cells from control uninfected cows. The number of apoptosis related genes present in our initial analysis prompted us to return to the microarray data and reduce our stringency of gene selection, requiring a *P*-value less than 0.15 instead of 0.05. As expected, this less stringent requirement highlighted many more genes, including those encoding CD95 and TNFR1 as being differentially expressed in PBMCs from infected cows relative to PBMCs from controls. Subsequent Q-RT-PCR verified that, indeed, as predicted by the microarray results, expression of the genes encoding CD95 and TNFR1 were expressed at significantly higher levels in PBMCs from *M. paratuberculosis*-infected cows than in similar cells from uninfected controls. Our novel results indicate that the gene-expression profile of PBMCs from *M. paratuberculosis*-infected cows is highly pro-apoptotic. These results are in agreement with recent studies indicating that, upon stimulation with *M. paratuberculosis*, PBMCs from infected cows, but not from uninfected controls, rapidly enter an apoptotic program (Grell et al., in preparation).

Finally, while results presented in this report are significant in that they point to several novel hypotheses regarding interactions between *M. paratuberculosis* and the host immune system, they also have profound implications for potential diagnostics aimed at detecting host responses to *M. paratuberculosis*. This fact, combined with recent progress in using gene-expression ''signatures'' to predict stage and metastatic potential of various human tumors (Alizadeh et al., 2001; Beer et al., 2002; Best et al., 2003), suggests that it may be possible to develop gene-expression assays for various metabolic and infectious diseases. To this end, we have recently completed a study using BOTL-5 microarrays with ~1500 genes to analyze gene-expression patterns in PBMCs from 10 *M. paratuberculosis*-infected cows compared as described in this report with 7 separate control uninfected cows and further validated using 17 infected and 9 control cows. These studies reveal that, indeed, there are consistent and detectable gene-expression differences in PBMCs from *M. paratuberculosis*-infected cows that may form an *M. paratuberculosis*-infection gene-expression ''signature'' (Skovgaard et al., in preparation). One caveat to this is that our work with a long-term chronic infection may not be directly applicable to more rapid infections, such as those with *E. coli* spp., *Salmonella* spp., or various viruses. However, work presented in another article in this volume suggests that these infections too leave a specific signature in the gene-expression patterns of host immune cells (Wilson et al., 2005).

Acknowledgements

The authors wish to acknowledge the outstanding technical support of Chris Colvin and Sue Sipkovsky. The assistance of Susanne Nedergård Grell and Gregers Jungersen from the Danish Institute for Food and Veterinary Research is also acknowledged. The authors wish to express their appreciation to Dr. Jeanne Burton for many helpful discussions and critical review of this manuscript. We also acknowledge the generous financial support of the College of Agriculture and Natural Science, the Michigan Agricultural Experiment Station, the Office of the Vice President for Research and Graduate Studies at Michigan State University, the MSU Foundation. Additional support for this project was provided by the USDA IFAFS Grant number 2001-52100-11211, The National Research Agency of Denmark (Grant no. 23-01-0163), and USDA-APHIS Veterinary Services Grant number 03-9100-0794-GR.

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